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Inhibition of nitric oxide production in lipopolysaccharide-stimulated RAW264.7 macrophage cells by lignans isolated from *Euonymus alatus* leaves and twigs

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ABSTRACT

The 80% methanolic extract of *Euonymus alatus* leaves and twigs afforded three new lignans, (−)-*threo*-4,9,4′,9′-tetrahydroxy-3,7,3′,5′-tetramethoxy-8-*O*-8′-neolignan (**1**), (−)-*threo*-4,9,4′,9′-tetrahydroxy-3,5,7,3′-tetramethoxy-8-*O*-8′-neolignan (**2**), (7*R*,8*R*,7′*R*)-(+)-lyoniresinol (**3**), together with seventeen known lignans (**4**–**20**). The structures of **1**–**20** were elucidated by extensive 1D and 2D spectroscopic methods including ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY, HMQC, HMBC and NOESY. All the isolated compounds except for dilignans (**19** and **20**) significantly inhibited nitric oxide production in lipopolysaccharide-stimulated RAW264.7 cells.

Nitric oxide (NO) is a gaseous molecule that transmits signals and regulates various physiological and pathological responses depending on the relative concentration of NO and the surrounding milieu in which NO is produced.[1] Regardless of pivotal roles of NO in the regulation of physiological functions, it also has been related to a number of pathologies, especially in inflammation and sepsis.[2] Chronic inflammation is an undesirable phenomenon which ultimately lead to developments of inflammatory diseases, such as rheumatoid arthritis, bronchitis, gastritis, multiple sclerosis, and inflammatory bowel disease.[3]

In the course of searching for compounds that inhibit NO production from natural sources using lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells as an in vitro assay system, it was found that an 80% methanolic extract of the leaves and twigs of *Euonymus alatus* (Thunb.) Sieb. significantly inhibited LPS-induced NO production in RAW264.7 cells. *E. alatus* is a deciduous tree of Celastraceae family. This tree is commonly known as winged euonymus or 'gui-jun woo', and has been used in oriental traditional medicine to regulate blood circulation, to relieve pain, to eliminate stagnant blood and to treat dysmenorrheal. Recently, antihyperglycaemic, hyperlipidaemic and immune-stimulating activities of *E. alatus* have been reported.[4–6] Known constituents of *E. alatus* include sesquiterpenes, sesquiterpene alkaloids, triterpenes, flavonoids and phenolic compounds.[7–9] To date, however, there has been no report on anti-inflammatory constituents of this plant. Thus, we have attempted to isolate compounds having anti-inflammatory activity from the methanolic extract of *E. alatua* using bioactivity-guided fractionation technique.

The leaves and twigs of *E. alatus* were collected in Nambu forest of Seoul National University, Beagwoon Mountain, Korea in September 2007 and authenticated by Dr. Jong Hee Park, Professor of Pusan National University. The air-dried plant material (15 kg) was extracted three times with 80% MeOH in an ultrasonic apparatus. Removal of the solvent in vacuo yielded a methanolic extract (1.2 kg). The methanolic extract was then suspended in distilled water and partitioned successively with $CHCl_3$, EtOAc, and *n*-BuOH. Each fraction was evaluated for its inhibitory activity on NO production in LPS-stimulated RAW264.7 cells. Among the four fractions, the $CHCl_3$ and EtOAc fractions, which significantly showed the inhibitory activities on NO production were used for the isolation of active compounds. Thirteen neolignans (**1**, **2**, **4**–**14**), three lignans (**3**, **15**, **16**), two sesquilignans (**17**, **18**) and two dilignans (**19**, **20**) were yielded. The known compounds were identified as (+)-simulanol (**4**),[11] (+)-dehydrodiconiferyl alcohol (**5**),[10] (−)-simulanol (**6**),[11] (−)-dehydrodiconiferyl alcohol (**7**),[10] (+)-dihydrodehyrodiconiferyl alcohol (**8**),[12,13] 7*R*,8*S*-guaiacylglycerol-8-*O*-4′-(coniferyl alcohol) ether (**9**),[14] 7*S*,8*R*-guaiacylglycerol-8-*O*-4′-(coniferyl alcohol) ether (**10**),[14] 7*S*,8*R*-syringylglycerol -8-*O*-4′-(synapyl alcohol) ether (**11**),[14] 7*S*,8*S*-guaiacylglycerol-8-*O*-4′-(synapyl alcohol) ether (**12**),[14] 7*S*,8*S*-4,9,9′-trihydroxy-3,3′-dimethoxy-8-*O*-4′-neolignan (**13**),[15] 7*R*,8*R*-4,9,9′-trihydroxy-3,

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3′-dimethoxy-8-*O*-4′-neolignan (**14**),[15] (+)-syringaresinol (**15**),[16] De-4′-methylyangabin (**16**),[17] hedyotol C (**17**),[18] *threo*-buddlenol B (**18**),[19] hedyotisol C (**19**),[20] hedyotisol B (**20**)[20] respectively, by comparison of spectroscopic data with those previously reported (Fig. 1).

Compound **1** was obtained as colorless syrup. The molecular formula of **1** was determined to be $C_{22}H_{30}O_9$ from HREIMS. The IR spectrum indicated that **1** possesses hydroxy (3400 cm^{-1}), phenyl (1600, 1460 cm^{-1}), and ether (1225 cm^{-1}) functional groups. The ^1H NMR spectrum revealed the signals for aromatic protons of an ABX system [δ_H 6.90 (1H, d, J = 1.5 Hz, H-2), 6.76 (1H, d, J = 10.0 Hz, H-5), 6.74 (1H, dd, J = 10.0, 1.5 Hz, H-6)], one AB system [δ_H 6.51 (1H, s, H-2′), 6.51 (1H, s, H-6′)], and four methoxy groups [δ_H 3.84 (3H, s, OCH_3-3), 3.82 (6H, s, OCH_3-3′, 5′), 3.20 (3H, s, OCH_3-7)] (Table 1). The ^1H–^1H COSY correlations in combination with HMQC spectroscopic data also revealed the presence of 1,2,3-propanetriol [δ_H 4.06 (H-7), 3.65 (H-8), 3.40 (H-9a), 3.30 (H-9b); δ_C 86.4 (C-7), 77.8 (C-8), 64.7 (C-9)] and 1,2-propanediol [δ_H 2.72 (C-7′a), 2.58 (C-7′a), 3.79 (C-8′), 3.48 (C-9′a), 3.44 (C-9′b); δ_C 41.7 (C-7′), 75.4 (C-8′), 67.3 (C-9′)]. The attachment of these groups to aromatic ring was evidenced by HMBC correlations between H-7/C-2, C-6 and H-7′/C-2′, C-6′ (Fig. 2). The cross peaks observed in NOESY spectrum between H-2 and 3-OCH_3, and between H-2′, 6′ and 3′, 5′-OCH_3 placed the methoxy groups at C-3, C-3′ and C-5′.

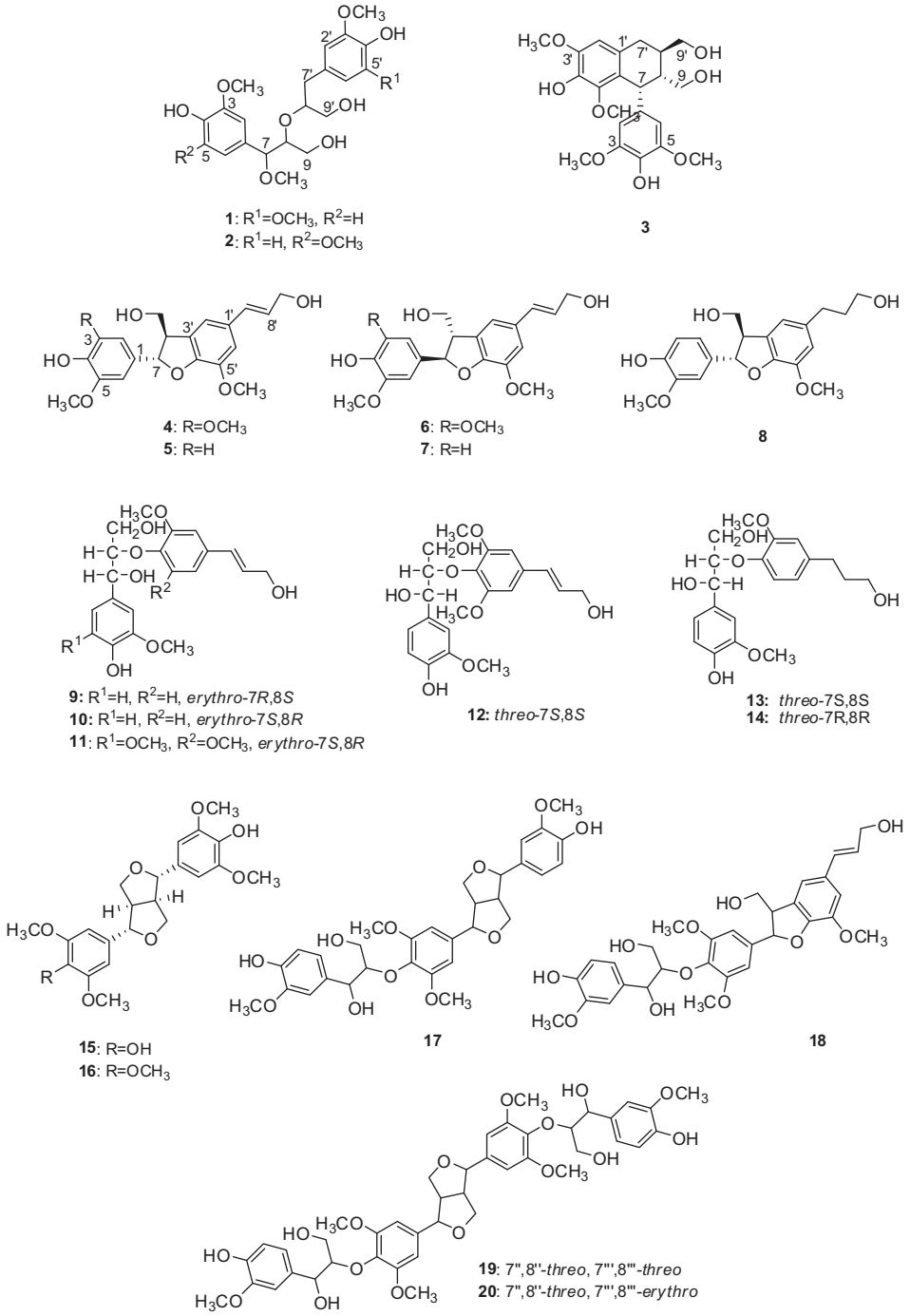

Figure 1. Structures of compounds **1**–**20** isolated from the leaves and twigs of *E. alatus*.

Table 1
NMR spectroscopic data (500 MHz, CD_3OD) for compounds **1**–**3**[a]

Pos.	1		2		3	
	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.
1		132.3, C		131.5, C		140.1, C
2	6.90 d (1.5)	112.1, CH	6.60 s	106.6, CH	6.37 s	107.7, CH
3		149.9, C		150.1, C		149.8, C
4		148.3, C		135.1, C		135.3, C
5	6.76 d (10.0)	116.8, CH		150.1, C		149.8, C
6	6.74 dd (10.0, 1.5)	122.3, CH	6.60 s	106.6, CH	6.37 s	107.7, CH
7	4.06 d (6.8)	86.4, CH	4.07 d (6.75)	86.6, CH	4.30 d (5.5)	43.1, CH
8	3.65 m	77.8, CH	3.65 m	77.8, CH	1.93–1.98 m	50.6, CH
9	3.40, 3.30	64.7, CH_2	3.42, 3.29	65.1, CH_2	3.46–3.50 overlapped	65.0, CH_2
					3.59 dd (10.8, 5.0)	
1′		131.6, C		132.5, C		130.9, C
2′	6.51 s	108.5, CH	6.81 d (1.7)	114.9, CH	6.57 s	108.8, CH
3′		149.9, C		149.8, C		139.7, C
4′		135.0, C		147.0, C		149.5, C
5′		149.9, C	6.70 d (8.0)	116.8, CH		148.5, C
6′	6.51 s	108.5, CH	6.65 dd (8.0, 1.7)	123.6, CH		127.0, C
7′	2.72 dd (13.7, 5.5)	41.7, CH_2	2.72 dd (13.8, 5.7)	41.3, CH_2	2.56 dd (15.0, 11.4)	34.4, CH_2
	2.58 dd (13.7, 7.5)		2.58 dd (13.8, 7.4)		2.68 dd (15.0, 4.8)	
8′	3.79 m	75.4, CH	3.76 m	75.4, CH	1.58–1.65 m	41.7, CH
9′	3.48 dd (11.1, 4.4)	67.3, CH_2	3.48 dd (11.2, 4.4)	67.3, CH_2	3.49 d (5.2)	67.6, CH_2
	3.44 overlapped		3.42 dd (11.2, 6.1)			
3-OMe	3.84 s	57.1, CH_3	3.82 s	57.5, CH_3	3.73 s	57.6, CH_3
5-OMe			3.82 s	57.5, CH_3	3.73 s	56.7, CH_3
7-OMe	3.20 s	57.6, CH_3	3.22 s	57.7, CH_3		
3′-OMe	3.82 s	57.5, CH_3	3.81 s	57.1, CH_3		
4′-OMe					3.85 s	57.4, CH_3
5′-OMe	3.82 s	57.5, CH_3			3.37 s	60.9, CH_3

[a] All assignments were made by extensive analysis of 1D and 2D NMR.

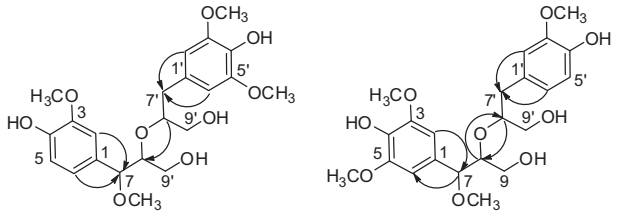

Figure 2. The selected HMBC correlations in compounds **1** and **2**.

On the basis of these data, it was deduced that **1** contains guaiacylglycerol and syringyl moieties in its structure. Determination of final structure of **1** was determined by the cross peak from C-8′ to H-8 in HMBC spectrum indicating the 8-*O*-8' linkage of two partial structures. The relative configuration of H-7/H-8 was assignable from the coupling constant of H-7 and H-8 (J = 6.8 Hz) indicating that glycerol moiety of **1** possess a *threo* relative configuration.[15] From the negative optical rotations, the structure of **1** was determined as (−)-*threo*-4,9,4′,9′-tetrahydroxy-3,7,3′,5′-tetramethoxy-8-*O*-8′-neolignan. Absolute configuration of **1** has not been fully determined because obtained amount of **1** was too small.

Compound **2** was also obtained as colorless syrup. The ^1H and ^{13}C NMR spectroscopic data of **2** were similar to those of **1**, except for the location of methoxy groups (Table 1). In NOESY spectrum, the cross peaks between H-2, 6 and 3, 5-OCH_3, and between H-2′ and 3′-OCH_3 were observed suggesting that **2** has syringylglycerol and guaiacyl moieties in its structure. HMBC correlations between C-8′/H-8 and C-8/H-8′ also suggested the 8-*O*-8′ linkage of two partial structures (Fig. 2). The relative configuration of H-7/H-8 was assignable from the coupling constant of H-7 and H-8 (J = 6.7 Hz) indicating that glycerol moiety of **2** also possess a *threo* relative configuration.[15] The structure of **2** was further confirmed by additional 2D spectroscopic data including HMQC and HMBC. On the basis of above data **2** was determined as (−)-*threo*-4,9,4′,9′-tetrahydroxy-3,5,7,3′-tetramethoxy-8-*O*-8′-neolignan.

Compound **3** was obtained as whitish amorphous powder. Its molecular formula was assigned as $C_{22}H_{28}O_8$ by HREIMS. The ^1H NMR spectrum revealed the signals for an symmetric 1,3,4,5-tetrasubstituted aromatic protons of AB system at δ_H 6.37 (2H, s, H-2, 6), an aromatic singlet at δ_H 6.57 (1H, H-2′) and two methoxy groups [δ_H 3.85 (3H, s, OCH_3-4′), 3.73 (6H, s, OCH_3-3, 5), 3.37 (3H, s, OCH_3-5′)]. ^1H NMR spectrum also showed the signals for three methine protons, one methylene proton and two oxymethylene protons (Table 1). ^1H–^1H COSY spectrum revealed their linkage as C^7H-$C^8H(C^9H_2O)$-$C^{8\prime}H(C^{9\prime}H_2O)$-$C^{7\prime}H_2$, which was confirmed by HMBC experiment. Further HMBC correlations between H-7′/C-2′, H-7/C-2, 6, 5′ strongly suggested a lignan structure of **3**. The cross peaks observed in NOESY spectrum between H-2′ and OCH_3-3′, and between H-2, 6 and OCH_3-3, 5 located the methoxy groups at C-2′ and C-2, 6, respectively. The location of the remained methoxy group was determined at C-5′ from HMBC correlations between H-7/C-5′ and OCH_3-5′/C-5′. These evidences suggested that the structure of **3** is an enantiomer of lyoniresinol.[21] The relative configuration of **3** was determined by the correlation peaks observed in HMBC and NOESY spectroscopic data. The coupling constant of H-7′ [δ_H 2.56 (1H, dd, J = 15.0, 11.4 Hz, H-7′a), 2.68 (1H, dd, J = 15.0, 4.8 Hz, Hb-7′b)] suggested that H-8 is α-oriented. The small coupling constant of H-7 (J = 5.5) was also indicative of *cis* orientation of H-7 and H-8. Finally, the strong NOESY correlations were observed between H-7′ and H-9′b, and between H-7 and H-9′a. From the positive optical rotations ($[\alpha]_D^{25}$ +17.5 in CH_3OH) of **3**, the absolute configuration of **3** was determined as 7*R*, 8*R*, 7′*R*, and ^{13}C NMR chemical shifts of C-7, 8, 9, 7′, 8′, 9′ in **3** was also agreed to the characteristic values of 7*R*, 8*R*, 7′*R*-cyclolignans.[22] Consequently, the structure of compound **3** was determined to be (7*R*,8*R*,7′*R*)-(+)-lyoniresinol, as shown in Figure 1.

All compounds isolated herein are reported for the first time from this plant.

Inhibitory effects of compounds **1**–**20** on LPS-induced NO production in RAW264.7 cells were evaluated using the Griess reaction (Table 2). The treatment of RAW264.7 cells with LPS resulted in a significant increment of nitrite concentration in the medium as compared to non-treated control. All the isolated compounds except for dilignans (**19**, **20**) showed the significant inhibitory activity on LPS-induced NO production in RAW264.7 cells. Among the isolated lignans, compounds **4**–**8** which belong to dihydrobenzofuran neolignan showed the most potent inhibitory activity with IC_{50} values of 8.5–12.8 μM (Table 2). The presence of a phenylpropanoid group in compound **18** slightly declined the inhibitory activity on NO production. Compounds **15** and **16**, dioxabicyclo[3,3,0] lignans, were found to be more potent than positive control, L-NAME with IC_{50} values of 15–20 μM. The inhibitory activity of compound **17** which possess phenylpropanoid group in its molecule was also found to decrease compared to those of compounds **15** and **16**. From the IC_{50} values of **15**–**18**, the basal skeleton of lignan seemed to be crucial for inhibitory activity on NO production in RAW264.7 macrophage cells.

Lignans have been reported for various pharmacological activities, especially on the inflammatory responses and the progress of cancer. Syringaresinol and its glycosides have been reported to have the significant cytotoxic activities in various tumor cell lines;[23–25] Lyoniresinol, a typical aryltetralin lignan, also exhibited antioxidant and antimutagenic effects.[26,27] Hence, synthetic chemistry and biotechnological methods have been developed to synthesize a variety of lyoniresinol derivatives.[28,29] Rahman et al.[30] proposed that (±)-syringaresinol might be the predicted intermediates in biosynthesis of lyoniresinol contained in *Lyonia ovalifolia*. On the other hand, the research on anti-inflammatory or anti-cancer activities of neolignans are poorly performed until now and limited to those of magnolol and honokiol. Magnolol was reported to suppress inflammatory process in endothelial cells[31] and to attenuate the production of leukotriene in rat bosaphilic leukemia-2H3 cells[32] and the formation of local edema.[33] Honokiol inhibited effectively the production of NO and tumor necrosis factor-alpha (TNF-α) in LPS-activated RAW264.7 cells,[34] and 4-*O*-methylhonokiol was reported to present anti-inflammatory effects through NF-κB pathway.[35] In the present study, lignans, neolignans, sesquilignans isolated from *E. alatus* leaves and twigs significantly inhibited NO production in LPS-stimulated RAW264.7 macrophage cells. To our knowledge, all compounds isolated herein are reported for the first time from this plant. The isolated compounds are thought to partially contribute to anti-inflammatory activity of *E. alatus*, and expected to possess beneficial therapeutic potential against various inflammation-related diseases.

Table 2
Inhibitory effects of compounds **1**–**20** isolated from *E. alatus* leaves and twigs on NO production induced by LPS in RAW264.7 cells

Compounds	IC_{50} (μM)	Compounds	IC_{50} (μM)
1	44.3 ± 1.7*	11	57.9 ± 5.4*
2	41.9 ± 2.0*	12	58.2 ± 3.7*
3	55.5 ± 1.3*	13	69.2 ± 9.6*
4	11.7 ± 1.2**	14	63.4 ± 5.8*
5	9.3 ± 1.4**	15	14.8 ± 2.5**
6	12.8 ± 2.0**	16	15.8 ± 1.6**
7	8.5 ± 0.8**	17	21.4 ± 2.0**
8	9.8 ± 2.0**	18	21.3 ± 3.3**
9	63.6 ± 4.1*	19	>100
10	66.4 ± 3.2*	20	>100
L-NAME	48.5 ± 2.3*		

RAW 264.7 cells were pre-treated with each compound for 1 h before exposure to LPS for 24 h. The concentration of nitrite in culture medium was measured using Griess assay (methods are described in Supplementary data). The values shown are the mean ± SD of data from three independent experiments. Significant compared with LPS alone, *P <0.01, **P <0.001. L-NAME (L-Nitro-Arginine Methyl Ester), the NOS inhibitor, was used as positive control.

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Supplementary data

Supplementary data (experimental information, physicochemical and NMR spectroscopic data of compounds **1**–**3**) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.02.102.

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